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14. ABSTRACT The goal of this project is to characterize the role of NF-kappaB2/p52 in the aberrant activation of AR signaling in castrationresistant prostate cancer. Our preliminary data demonstrate that NF-kappaB2/p52 is expressed at high levels in prostate canceand that overexpression of NF-kB2/p52 facilitates castration resistant prostate cancer progression by activation of AR signalingand rescue of cancer cells from apoptotic death induced by androgen deprivation. Our hypothesis is that NF-kappaB2/p52activates the AR and protects prostate cancer cells from apoptotic cell death induced by androgen deprivation therapy, leadingto the development of castration resistance prostate cancer. The specific aims are: 1. To determine the role of p52 activation inandrogen responsiveness and progression of castration resistant prostate cancer. 2. To determine the effect of expression op52 on the development and progression of prostate cancer in a transgenic mouse model. 3. To determine the mechanisms oAR activation by p52.					
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Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusions.....	4
References.....	4

Introduction

The goal of this project is to characterize the role of NF- κ B2/p52 in the aberrant activation of AR signaling in castration-resistant prostate cancer. The growth of prostate cancer is initially dependent on androgen and can be effectively treated by androgen-deprivation therapy. However, androgen-deprivation therapy only causes a temporary regression of prostate cancer, as all tumors will eventually progress to refractory to hormonal therapy (castration resistant prostate cancer). Androgen signaling through androgen receptor (AR) plays an important role not only in maintaining the function of the prostate, but also in promoting the development of androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer. Our preliminary data demonstrate that NF- κ B2/p52 is expressed at high levels in prostate cancer and that overexpression of NF- κ B2/p52 facilitates castration resistant prostate cancer progression by activation of AR signaling and rescue of cancer cells from apoptotic death induced by androgen deprivation.

Progress Report for the Year 15 Jul 2010-14 Jul 2011:

We have made significant progress in Task 1 (i.e., Determination of whether knockdown of endogenous p52 expression in androgen insensitive C4-2 and LNCaP-IL6+ cells can block tumor growth).

Downregulation of p52 inhibits prostate tumor growth

We obtained retroviral vectors (pSM2C) encoding shRNA against NF-kappaB2 from Open Biosystems. The vectors were packaged into retroviral particles by transfection into Phoenix Amphi retroviral packaging cells (Allele Biosciences) along with packaging plasmids. Control retroviruses encoding the empty vector were also generated. The titer of the retroviral supernatant was determined and the ability of the retroviruses to downregulate expression levels of NF-kappaB2 was verified by Western blotting. These retroviral particles were used in *in vivo* assays to determine the ability of knockdown of p52 to block tumor growth of C4-2B prostate cancer cells.

2x10⁶ cells/flank C4-2B cells were injected sub-cutaneously into both flanks of male nude mice. Tumor growth was monitored twice weekly with tumor volume measurement using calipers. Once the tumors reached 0.5 cm³, 100 μ l (10¹⁰ particles) of the concentrated retroviral particles encoding either shRNA against p52 or control were injected intratumorally. Tumor growth was monitored twice weekly. We found that injection of retroviral particles encoding p52 shRNA abolished tumor growth significantly compared to the control retroviruses (**Fig. 1A**). At the end of 3 weeks, the experiment was terminated since the tumors injected with control retroviruses reached the maximum limit of tumor size allowed by the IACUC. Tumors were excised and blood was collected for measurement of PSA levels in the sera by ELISA. As shown in **Fig. 1B**, tumors injected with shRNA against p52 produced lower levels of PSA compared to the controls. Total protein extracts were prepared from the tumor tissues and expression levels of a few NF-kappaB2 target genes were examined by Western blotting. As shown in **Fig. 1C**, p52 levels were downregulated significantly in tumors injected with p52 shRNA. Expression levels of Cyclin D1, c-Myc and TWIST-2 were also found to be reduced in tumors injected with p52 shRNA. Total RNAs were also prepared from the tumor tissues and expression levels of AR, PSA and NKX3.1 were examined. Expression

levels of AR were not affected in tumors injected with p52 shRNA, while knockdown of p52 inhibited expression of PSA and NKX3.1, two typical target genes of the AR (Fig. 1D). These results show that downregulation of endogenous p52 expression *in vivo* may inhibit tumor growth and activation of the AR in prostate cancer cells.

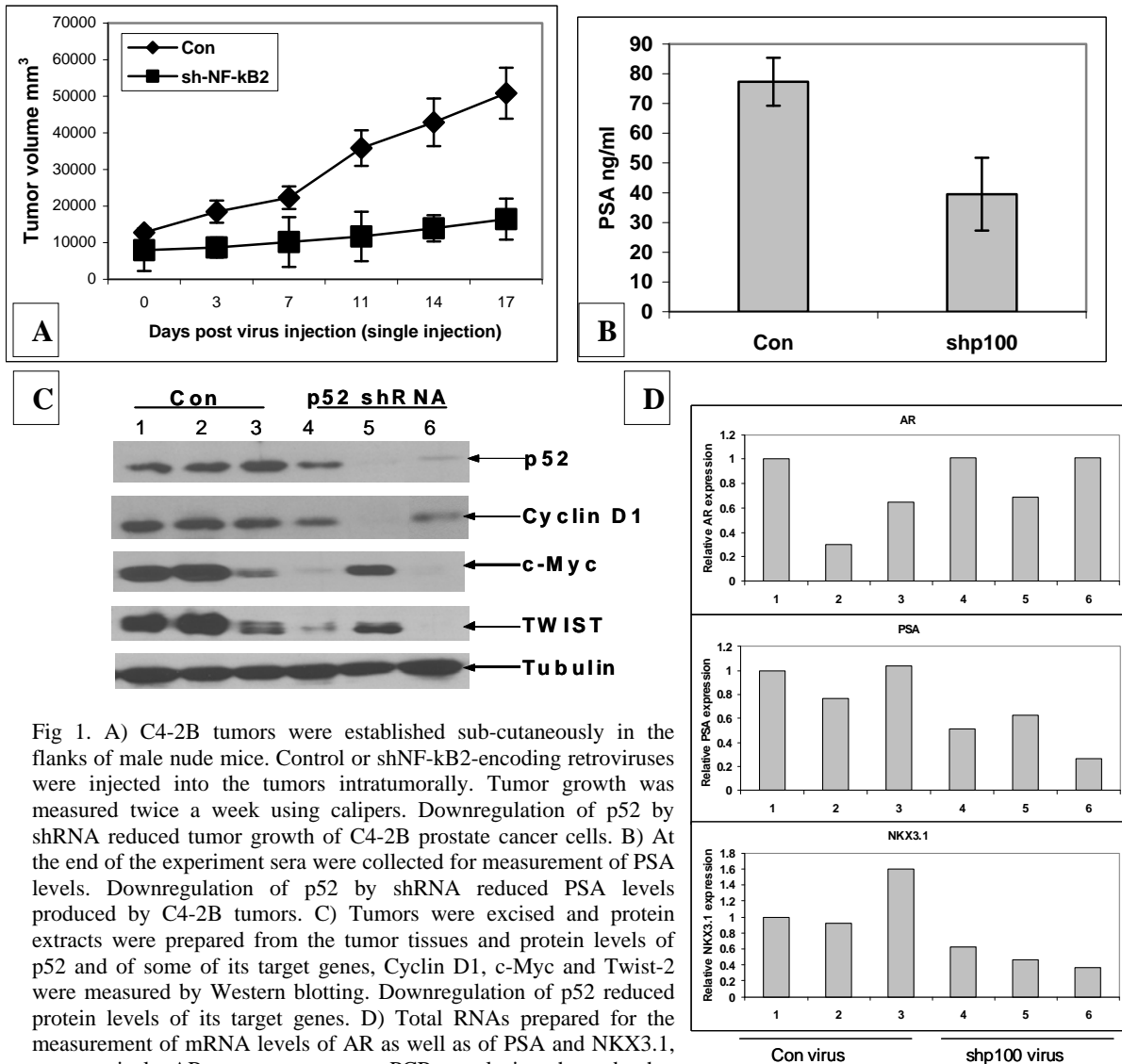


Fig 1. A) C4-2B tumors were established sub-cutaneously in the flanks of male nude mice. Control or shNF-kB2-encoding retroviruses were injected into the tumors intratumorally. Tumor growth was measured twice a week using calipers. Downregulation of p52 by shRNA reduced tumor growth of C4-2B prostate cancer cells. B) At the end of the experiment sera were collected for measurement of PSA levels. Downregulation of p52 by shRNA reduced PSA levels produced by C4-2B tumors. C) Tumors were excised and protein extracts were prepared from the tumor tissues and protein levels of p52 and of some of its target genes, Cyclin D1, c-Myc and Twist-2 were measured by Western blotting. Downregulation of p52 reduced protein levels of its target genes. D) Total RNAs prepared for the measurement of mRNA levels of AR as well as of PSA and NKX3.1, two typical AR target genes. qPCR analysis showed that downregulation of p52 reduced mRNA levels of PSA and NKX3.1, but not those of the AR.

We have made significant progress in Task 2 (i.e., determine the effect of expression of p52 on the development and progression of prostate cancer in a transgenic mouse model).

2a. Construction of promoter for prostate specific p52 expression

A pBluescript-based vector containing the ARR₂-PB promoter (pBS-ARR₂PB-IRES-HA-GFP) was obtained from Dr. Vasioukhin, Fred Hutchinson Cancer Center, Seattle, WA. As shown in Fig. 2, this vector contains the androgen-dependent probasin promoter

followed by the β -globin intron, an internal ribosome entry site (IRES) and GFP. The coding region of p52 was cloned in frame between NotI and EcoRI sites of this vector and the insert identity was confirmed by restriction digestion and sequencing. Expression of p52 was also confirmed by transfection into HEK293T cells and Western blotting. The excised insert was purified by Qiagen column purification and used for microinjection into B6D2F1 hybrid pronuclei. The microinjection was performed by the Mouse Biology Core at the University of California Davis.

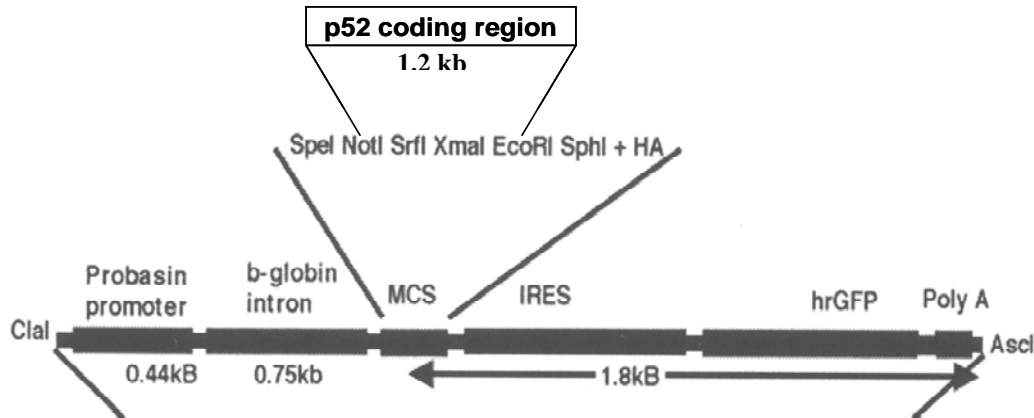


Fig. 2. Schematic diagram showing the region in the MCS where the coding region of p52 was cloned in frame with the probasin promoter, HA-tag and GFP.

2b. Generation of p52 transgenic mice

Microinjection produced 3 male and 3 female founders, which were mated to produce the F1 generation. 3 transgenic lines were generated and further breeding was performed to generate F2 generation. The founder males and some of the F1 generation males were reserved for aging to examine the role of p52 expression on the aging prostate. The expression of p52 in the mouse prostates was verified by PCR (**Fig. 3**). The breeding program results are summarized in the table below.

Future directions: We will continue the breeding program to verify the transmittance of the transgene upto 4-5 generations. This will be followed by examination of the prostates of male mice of different age groups to assess the effects of expression of p52 in the prostate and any phenotypic changes that may occur.

	Br Pair	Litters	# of progeny	# positive	# Male	# Female
Line 1	17/20	3	14	6	2	4
	F2	3	13	6	4	2
Line 2	10/30	3	29	21	8	13
	F2	6	45	22	10	12
Line 3	5/7	2	9	7	5	2

Table 1. Numbers of progeny obtained from the 3 breeding pairs of founders are summarized in the table.

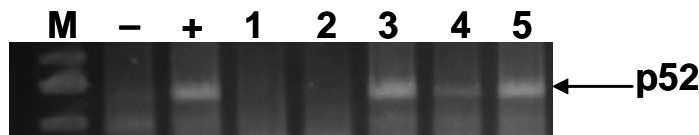


Fig. 3. Expression of p52 was verified by PCR in the mouse prostates. M=Marker; - = negative control; + = positive control

Key Research Accomplishments:

We have:

- ◆ demonstrated that downregulation of endogenous p52 inhibits tumor growth of human prostate cancer cells *in vivo*.
- ◆ generated a transgenic construct for prostate-specific expression of p52 using the ARR2PB promoter.
- ◆ successfully generated transgenic mice expressing p52 in the prostate under the control of the ARR2PB promoter.
- ◆ successfully generated F1 and F2 generations of the transgenic mice and verified transmittance of the p52 transgene in mice.

Reportable outcomes:

Abstracts:

- | | |
|------|---|
| 2011 | Regulation of intracrine androgen synthesis by NF-kappaB2/p52, AUA Annual Meeting, Washington, DC |
| 2011 | Regulation of intracrine androgen synthesis by NF-kappaB2/p52, AACR Annual Meeting, Orlando, FL |

Conclusions:

- ◆ We demonstrated that knockdown of endogenous p52 inhibits tumor growth of human prostate cancer cells.
- ◆ We generated transgenic mice expressing p52 specifically in the prostate under the control of the ARR2PB promoter.

References:

1. Nadiminty N, Chun JY, Lou W, Lin X, Gao AC (2009) NF-kappaB2/p52 enhances androgen-independent growth of human LNCaP cells via protection from apoptotic cell death and cell cycle arrest induced by androgen deprivation. *Prostate* 68(16): 1725-33.
2. Nadiminty N, Dutt S, Tepper C, Gao AC (2010) Microarray analysis reveals potential target genes of NF-kappaB2/p52 in LNCaP prostate cancer cells. *Prostate* 70(3): 276-87.
3. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung HJ, Evans CP, Zhou Q, Gao AC (2010). Aberrant activation of the androgen receptor by NF-kappaB2/p52 in prostate cancer cells. *Cancer Res* 70(8): 3309-19.

Progress Report for the year Jul 2009-Jul 2010:

Key Research Accomplishments

We demonstrated that:

- Downregulation of p52 inhibits growth of prostate cancer cells
- Downregulation of p52 reduces expression of AR target genes like PSA and NKX3.1
- p52 induces nuclear translocation of AR in androgen-deprived conditions
- p52 induces AR DNA binding in a castration-resistant manner
- p52 induces recruitment of co-activators like p300 to the AR transcriptional complex in androgen-depleted conditions
- p52 activity is dependent on AR expression
- p52 regulates the expression of several genes involved in cell survival, angiogenesis and metastasis.

Reportable outcomes

Publications

1. Nadiminty N, Dutt S, Tepper C, Gao AC (2010) Microarray analysis reveals potential target genes of NF-kappaB2/p52 in LNCaP prostate cancer cells. *Prostate* 70(3): 276-87.
2. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung HJ, Evans CP, Zhou Q, Gao AC (2010) Aberrant activation of the androgen receptor by NF-kappaB2/p52 in prostate cancer cells. *Cancer Res* 70(8): 3309-19.

Abstracts

- | | |
|------|--|
| 2010 | April, Aberrant activation of the androgen receptor by NF-kappaB2/p52, Annual Meeting, AACR, Washington DC. |
| 2010 | June, Aberrant activation of the androgen receptor by NF-kappaB2/p52, AUA Annual Meeting, San Francisco, CA. |

Conclusions

- We demonstrated that p52 activates AR signaling.
- p52 promotes castration-resistant growth of androgen sensitive LNCaP cells *in vitro* and *in vivo*.

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Presentation Abstract

Abstract Number: 4554

Presentation Title: Regulation of intracrine androgen synthesis by NF-kappaB2/p52

Presentation Time: Tuesday, Apr 05, 2011, 1:00 PM - 5:00 PM

Location: Exhibit Hall A4-C, Poster Section 29

Poster Section: 29

Poster Board Number: 23

Author Block: Nagalakshmi Nadiminty, Ramakumar Tummala, Jae Yeon Chun, Christopher P. Evans, Allen C. Gao. UC Davis, Sacramento, CA

Abstract Body: Introduction: Benign prostatic hyperplasia and the initial stages of prostate cancer (CaP) exhibit androgen dependence, but androgen ablation results only in temporary regression of CaP, with progression to castration-resistant prostate cancer (CRPC). Androgen receptor (AR) signaling remains active in CRPC due to aberrant activation of the AR. Synthesis of intracrine androgens has emerged as one of the mechanisms by which AR is activated in CRPC after androgen ablation. Even though levels of androgen biosynthetic enzymes have been shown to be elevated in CRPC, their regulation is not completely understood. In this study, we examined the role of NF-kappaB2/p52 in intracrine androgen synthesis and castration-resistant progression of CaP. Methods: Expression levels of androgen biosynthetic enzymes were measured in CaP cells with or without expression of NF-kappaB2/p52 using real-time RT-PCR and western blotting. Regulation by NF-kappaB2/p52 was examined using luciferase reporter assays and plasmids containing regulatory elements of androgen biosynthetic enzymes. Intracrine levels of androgens were measured using EIA in tumors obtained from castrated mice. Results: Expression levels of androgen biosynthetic enzymes including AKR1C3, CYP17A1, HSD3B2, and SRD5A1 were found to be elevated in CaP cells expressing NF-kappaB2/p52. Luciferase assays showed that NF-kappaB2/p52 regulates their expression directly by binding to their promoters and inducing transcription. The levels of total testosterone in CaP cells expressing NF-kappaB2/p52 were approximately 3-fold higher than control cells as measured using EIA. Intraprostatic androgen levels were found to be at 1002 ± 232 pg/g tissue, compared to 377.8 ± 105 pg/g tissue in control tumors obtained by orthotopic implantation of CaP cells expressing NF-kappaB2/p52. These data suggest that CaP cells synthesize detectable levels of testosterone in the absence of exogenous steroid precursors and overexpression of NF-kappaB2/p52 can increase this process, possibly by enhancing the expression of genes encoding steroidogenic enzymes. Conclusions: Intraprostatic androgen synthesis in recurrent prostate tumors contributes significantly to resistance to androgen ablation and development of CRPC. NF-kappaB2/p52 regulates the expression levels of steroidogenic enzymes and thereby enhances synthesis of intracrine androgens and aberrant activation of the AR. Coupled with our previous studies, these data suggest that antagonizing NF-kappaB2/p52 signaling may prove beneficial in CRPC therapy.

American Association for Cancer Research
615 Chestnut St. 17th Floor
Philadelphia, PA 19106

Tuesday, May 17, 2011 8:00 AM-10:00 AM

Prostate Cancer: Basic Research

Moderated Poster

Source of Funding: NIH CA118887, CA 140468 and DOD PC080538.

1453: REGULATION OF INTRACRINE ANDROGEN SYNTHESIS BY NF-KAPPA B2/P52

nagalakshmi Nadiminty

Ramakumar Tummala

Jaeyeon Chun

Christopher Evans

Allen Gao

Sacramento, CA

INTRODUCTION AND OBJECTIVES: Benign prostatic hyperplasia and the initial stages of prostate cancer (CaP) exhibit androgen dependence, but androgen ablation results only in temporary regression of CaP, with progression to castration-resistant prostate cancer (CRPC). Androgen receptor (AR) signaling remains active in CRPC due to aberrant activation of the AR. Synthesis of intracrine androgens has emerged as one of the mechanisms by which AR is activated in CRPC after androgen ablation. Even though levels of androgen biosynthetic enzymes have been shown to be elevated in CRPC, their regulation is not completely understood. In this study, we examined the role of NF-kappaB2/p52 in intracrine androgen synthesis and castration-resistant progression of CaP. **METHODS:** Expression levels of androgen biosynthetic enzymes were measured in CaP cells with or without expression of NF-kappaB2/p52 using real-time RT-PCR and western blotting. Regulation by NF-kappaB2/p52 was examined using luciferase reporter assays and plasmids containing regulatory elements of androgen biosynthetic enzymes. Intracrine levels of androgens were measured using EIA in tumors obtained from castrated mice.

RESULTS:

Expression levels of androgen biosynthetic enzymes including AKR1C3, CYP17A1, HSD3B2, and SRD5A1 were found to be elevated in CaP cells expressing NF-kappaB2/p52. Luciferase assays showed that NF-kappaB2/p52 regulates their expression directly by binding to their promoters and inducing transcription. The levels of total testosterone in CaP cells expressing NF-kappaB2/p52 were approximately 3-fold higher than control cells as measured using EIA. Intraprostatic androgen levels were found to be at 1002 ± 232 pg/g tissue, compared to 377.8 ± 105 pg/g tissue in control tumors obtained by orthotopic implantation of CaP cells expressing NF-kappaB2/p52. These data suggest that CaP cells synthesize detectable levels of testosterone in the absence of exogenous steroid precursors and overexpression of NF-kappaB2/p52 can increase this process, possibly by enhancing the expression of genes encoding steroidogenic enzymes.

CONCLUSIONS:

Intraprostatic androgen synthesis in recurrent prostate tumors contributes significantly to resistance to androgen ablation and development of CRPC. NF-kappaB2/p52 regulates the expression levels of steroidogenic enzymes and thereby enhances synthesis of intracrine androgens and aberrant activation of the AR. Coupled with our previous studies, these data suggest that antagonizing NF-kappaB2/p52 signaling may prove beneficial in CRPC therapy.